Gene Transfer into Cultured Human Epidermis and its Transplantation onto Immunodeficient Mice: An Experimental Model for Somatic Gene Therapy

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To try epidermis as a target for somatic gene therapy we studied transfected primary human keratinocytes grown in culture and grafted onto athymic mice. We have developed a novel technique for grafting cultured epidermal sheets onto mice. First, the graft is placed on the dorsal muscle fascia underneath the mouse skin using the latter as a bandage. Secondly, the mouse skin above the graft is removed, which exposes the grafted skin to open air and thus stimulates terminal differentiation. A novel method for the discrimination between murine and human epidermal cells is also presented, employing *in situ* hybridization with human Alu repeated DNA sequences.

During monolayer culture the keratinocytes were lipofected with the gene for human growth hormone in an Epstein-Barr virus–based expression vector. The cells were allowed to develop a multilayered tissue for 5 d, secreting human growth hormone into the medium at a daily rate of at least 50 ng/cm² of tissue.

The transfected tissues were then grafted onto mice. We detected human growth hormone at levels of up to 2.6 ng/ml in mouse serum for 4 d, but later no human growth hormone could be found, although the transplants survived for months. To investigate the fate of the transfected cells in the transplanted tissue, we labeled them with the β-galactosidase reporter gene. The cells staining positive for X-gal were found exclusively in the most superficial differentiated layers at 7 d after transplantation. This may be the main reason why no human growth hormone is found in the mouse circulation at this time. *Key words:* grafting/nude mice/transfection/skin/culture. *J Invest Dermatol 103:391–394, 1994*

The transplantation of cultured keratinocytes onto athymic animals has numerous applications. It may be used to optimize the grafting techniques of severe burns [1], or to study events occurring during the terminal differentiation of normal or pathologic epidermis [2,3]. It may also be used in the context of gene therapy either to produce an organoid delivering or removing specific products systemically [4–7] or as a genetically improved graft that locally can deliver products like growth factors that could stimulate wound healing and the formation of a more developed dermis. Fenjves *et al* [4] showed that human apolipoprotein E (Apo E) was secreted into the blood of nude mice after the transplantation of cultured human primary keratinocytes. Using a carcinoma cell line transfected with the gene encoding human growth hormone (hGH), Teumer *et al* [5] showed it possible to detect this exogenous protein after the transplantation of the carcinoma cell sheet upside down under the mouse skin. The coagulation factor IX has also been transiently detected in the blood of nude mice after the grafting of retrovirally transduced human primary keratinocytes [6].

In the present study, we have transfected primary keratinocytes grown in monolayer. When the culture had been allowed to differentiate and stratify for 5 d, the resulting tissue was transplanted onto nude mice. The grafting technique described in this work was developed with the final goal to establish a permanent transplant in a normal position. The technique is simple and avoids the everted flap technique described by Barrandon *et al* [8] because this is difficult to perform in mice.

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Abbreviations: ApoE, apolipoprotein E; hGH, human growth hormone; K-SFM, keratinocyte serum free medium.

MATERIALS AND METHODS

Chemicals And Media PBS: Dulbecco's phosphate buffered saline without calcium and magnesium. PBS-ethylenediamine tetraacetic acid (EDTA): PBS, 0.3 mM EDTA. Dispase II (Neutral protease, Boehringer): 12.5 U/ml or 1.25 U/ml in Hanks' balanced salt solution (JRH, Seralab). Trypsin stock, 2.5% (JRH, Seralab). K-SFM: keratinocyte-serum-free medium including additives (Gibco, Life Technology). FBS: fetal bovine serum (Biocrom). Ep medium: Dulbecco's modified Eagle's medium (MEM) (Gibco, Life Technology), 15% FBS, EGF (epidermal growth factor, Collaborative Research Inc., Lexington, MA) 10 ng/ml, penicillin 250 units/ml, streptomycin 25 μg/ml, L-glutamin 580 mg/l, and hydrocortisone 0.4 μg/ml.

Cell Culture Human primary keratinocytes were isolated from specimens obtained from reconstructive surgery as follows. Dermal side was cleaned from fat. The epidermal side was superficially cut every 5 mm to ensure access of the dispase. The specimen was then washed in PBS and incubated in 12.5 U/ml dispase II overnight at 4°C. The epidermis was peeled from the dermis with a pincer and transferred to PBS-EDTA for 10 min at room temperature. Trypsin was added to a final concentration of 0.025% w/v and the tissue was incubated for a maximum of 20 min at 37°C. Cells were dissociated by shaking the tube. Trypsin action was stopped by

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adding ½ vol FBS and the cells were collected at 270 × *g*, 5–10 min. Cells were counted and resuspended in complete K-SFM and plated at a density of 2×10^5 cells/cm² in tissue-culture flasks.

Transfection When the cultures were about 85% confluent the cells were transfected by lipofection (DOTMA, Boehringer) according to the manufacturer. The hGH expression vector was constructed as described by Jensen *et al* [9] except that a 2.15-kb genomic fragment (BamHI/EcoRI) encoding the hGH was inserted in the site controlled by the SV40 promoter. The expression vector carrying the Lac Z gene was described by Jensen *et al* [10]. Epstein-Barr virus (EBV)-based expression vectors were chosen because of their potential ability to become established as replicating episomes in human lymphoblastoid cells and epithelial cell lines [11] and because they display pronounced stability in human keratinocytes, giving high expression for months post-transfection. DNA-DOTMA complexes were added for a maximum of 16 h. The cells were washed once in Ep medium, and then cultured in Ep medium to induce differentiation. The medium was changed every other day.

After 5 d the transfected tissue was incubated in dispase II 1.25 U/ml made up in Hanks' balanced salt solution. When the central part of the epidermal sheet was still loosely attached to the flask, the reaction was stopped by removing the dispase solution and adding Ep medium. The flask was placed on ice and the top cut away with a hot knife. Medium was removed and a piece of viscose rayon mesh (Melolite contact, Smith and Nephew Scandinavia A/S) 2 × 1.5 cm was placed on the epidermal sheet to support the transplant. The transplant was loosened from the substrate and transferred to a petri dish, placed tissue side down, and covered with a sterile cover slip. Drops of Hanks' balanced salt solution with 10 mM glucose were added to the edges of the cover slip in order to keep the transplant moist.

Transplantation Technique Step 1: female athymic mice NMRI-nu/nu were anesthesized with avertin by intraperitoneal injection. Skin was cleaned in 5% potassium iodide in 50% ethanol. A 4–5-cm fissure was cut laterally on the dorsal side of the mouse. Loose connective tissue between the skin and the fascia of the dorsal muscles was removed by blunt dissection. The transplant was placed directly on the fascia of the dorsal muscles, tissue side down. The fissure in the mouse skin was then closed with Histoacryl Blue (B.Braun Melsungen AG, Germany), and covered with Tegaderm (3M Company, USA). This allows the transplant to be established without any mechanical disturbance from bandages.

Step 2: on day 5, the mice were anesthesized with Hypnorm (Janssen Pharmaceutica N.V., Belgium) and Diazepam, Stesolid (DUMEX, A.L. Laboratory, NJ, USA) by intraperitoneal injection (because of induction of the cytochrome P-450 enzyme by avertin and related drugs it is recommended to use anesthesia based on morphine during step 2). The skin above the transplant was cleaned as described. The mouse skin was surgically removed to create a window exposing the entire transplant and a second piece of Melolite contact was placed over the wound area and kept in place with Tegaderm. The animals were kept at 26–28°C.

***In situ* Hybridization** After sacrificing the animal, the grafted area was removed and fixed overnight in 4% paraformaldehyde-PBS. Standard techniques for paraffin sections were used. Five-micrometer sections were collected onto slides coated with 3-amino-propyl-trietoxy-silane (Sigma, A-3648). Sections were deparaffinized in xylene, and dried from methanol. The tissue was treated with 100 μg/ml proteinase K (Merck, 24 568) at 37°C for 10–60 min in buffer composed of 100 mM Tris-HCl, pH 7.5, 50 mM EDTA. Postfixation was performed in 1% paraformaldehyde-PBS for 5 min, and the slides were then dried from methanol. Hybridization mixture was added and the sections were heated to 95°C for 7 min in a moist chamber. Sections were transferred to 42°C overnight. Hybridization mixture: 50% formamide, 5 × SSC, 0.1% sodium dodecylsulfate (SDS), 2 × Denhards solution, 10% dextran sulphate, 25 μg/ml salmon sperm DNA and 0.2 ng/μl probe, Blur 8 alu repeat [12] inserted into a PUC vector. The entire plasmid was used after nick translation with digoxigenin-labeled dUTP.

After incubation at 42°C, the sections were washed in 2 × SSC, 0.1% SDS, 2 × 5 min at room temperature, then 0.1 × SSC, 10 min at 42°C. The final wash was in 2 × SSC, 0.1% SDS for 5 min at room temperature.

Immunostaining was performed with an alkaline phosphatase–conjugated anti-digoxygenin Fab fragment, according to the manufacturer (Boehringer).

The histochemical visualization was achieved with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) [13].

β-Gal Staining of Grafts Tissue was prepared as described by Lemarchand *et al* [14]. The tissue was placed in fixative (2% formaldehyde, 0.2% glutaraldehyde in PBS) for 2 h and transfered into X-gal staining buffer [5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ with 200 μg/ml X-gal] at 37°C for 6 h. Tissue was then postfixed in fixative for 2 h and embedded in paraffin. Paraffin sections, prepared as described above, were counterstained in hematoxylin and eosin. About 600 sections were analyzed.

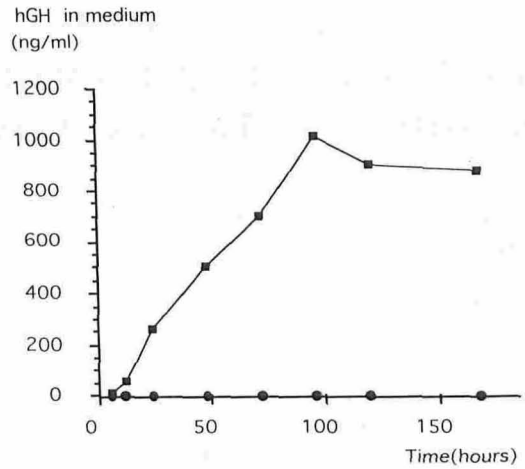

Figure 1. HGH secretion from tissue-cultured epidermal cells. Primary keratinocytes were transfected with vector without the hGH gene (●), or with the hGH gene inserted (■). Twenty percent of the medium was removed and replaced with new medium at each sample point. HGH was measured by enzyme-linked immunosorbent assay.

Enzyme-Linked Immunosorbent Assay Human growth hormone was determined directly in the growth medium or mouse serum in a dilution 1:10 or undiluted, respectively. An immunofluorometric assay was used (DELFIA, Wallac, Turku, Finland) employing two monoclonal antibodies directed at different sites. The mouse serum was obtained from tail blood. It should be noted that the mice are very small (about 20 g), and that this limits the amount of blood that can be harvested. We found that it was feasible to withdraw three portions of 120 μl whole blood during 10 d and that this was of no harm to the animals.

hGH Receptor Binding Assay A competition assay with IM-9 cells was performed according to de Meyts [15] and Lesniak *et al* [16]. IM-9 cells were washed and resuspended in assay buffer (100 mM Hepes, 120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 15 mM Na acetate, 10 mM Glucose, 1 mM EDTA, and 1% BSA, pH 7.4). The cells were incubated in 5 ml polypropylene tubes with 25 μl ^{125}I-hGH (22 kD/4.5×10^{-11} M) with or without unlabeled hGH (22 kD, 3×10^{-7} M), or with 25 μl dilutions of sample in a total volume of 250 μl and a cell density of 1.5×10^7 cells/ml. The tubes were incubated at 30°C for 90 min. Thereafter 200 μl of the cell mixture was layered on top of 50 μl di-N-butyl phthalate and the cells were centrifuged for 1 min in a microfuge at 9000 rpm to separate bound ^{125}I-hGH from unbound. The tip of the tube containing the cells was cut off and the radioactivity bound to the cells was measured in a gamma-counter. Specific binding is causing a drop in radioactivity with rising concentrations of hGH in the sample.

RESULTS

Primary cultures of adult human keratinocytes are readily transfected at high frequency using standard transfection methods. We have lipofected basal cell monolayers with an EBV-based expression vector [9] that carries a genomic insert of the gene encoding human growth hormone. By this protocol 5–10% transfected cells are achieved. The hGH gene is driven by an SV40 promoter and the daily secretion is at least 1250 ng of hGH from a 25 cm² culture flask with 5 ml medium **(Fig 1)**. The binding affinity of the secreted hGH was shown in the IM-9 receptor assays to be similar to standard hGH (see *Materials and Methods*). The high expression and secretion from the keratinocytes gave us the hope that these primary cells could be used as an organoid to deliver non-epidermal protein products into the blood stream. To test this possibility the transplanted cultures were allowed to differentiate and stratify. The epidermal sheets were then grafted onto nude mice. First the graft is placed on the dorsal fascia underneath the mouse skin and after 5 d, when the graft has taken, the overlying mouse skin is removed, allowing the graft to develop as an integument.

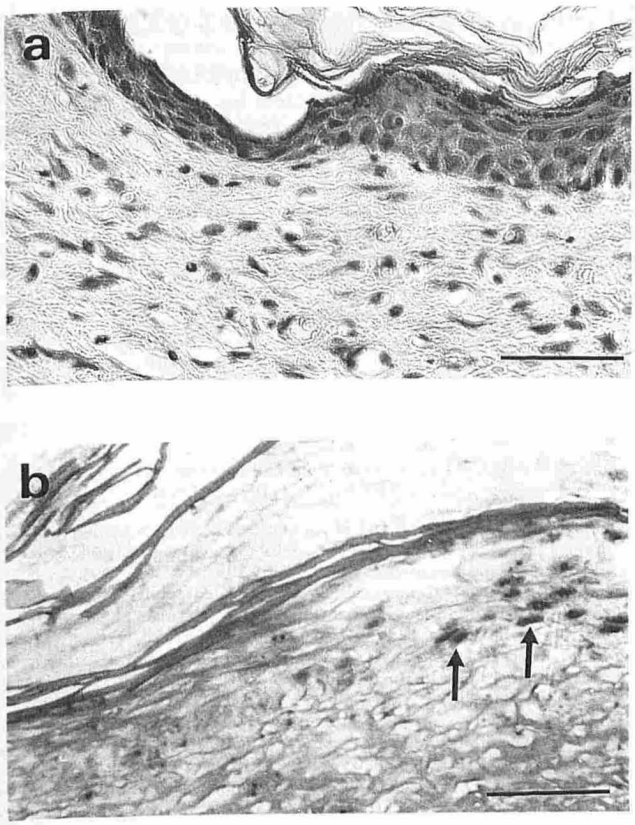

Figure 2. Human origin of transplanted keratinocytes. Tissue cultured transfected human primary keratinocytes at day 20 after transplantation onto nude mice. The murine/humane junction is shown. *a)* Hematoxylin/eosin staining. *b)* After *in situ* hybridization with a digoxigenin labeled probe against human Alu DNA sequences, the nuclei of the human epidermal cells are positive (some are marked with arrows) when immunostained with an alkaline phosphatase conjugated anti-digoxygenin Fab fragment. *Scale bars,* 50 μm.

The transplantation technique presented here has a take frequency close to 100% after the grafting step 1. After the second step, when the transplanted tissue is exposed to open air, the rate of success is dependent on the animal behavior and the efficiency of graft protection. About 50% of the grafts developed successfully as an integument that could survive for months. During closure of the wound after step 2, the graft area undergoes contraction to about 50% of its original size. At this point (about 2 weeks after step 2) the graft is no longer fixed to the muscle fascia, but can be moved quite easily, suggesting that the sheet of connective tissue that was removed during step 1 is reconstituted. This rapid formation of a murine "neo-dermis" is in good correlation with findings in burn patients after the grafting of autologous epidermis onto the muscle fascia [17]. After the initial contraction the graft seems stable for months. However, about a year after the transplantation the graft area becomes less conspicuous and eventually no human cells can be detected with certainty. **Figure 2** shows the graft site and the verification of the human origin by *in situ* hybridization‡ at day 20 after transplanting the transfected keratinocytes. Using this approach it is possible to detect any type of human tissue on the mouse, without the need for species-specific antibodies for the relevant tissue. Upon grafting of human full skin it is possible to show the human origin of cells in vascular endothelium, fibroblasts, hair follicles, glands, and epidermis several months post grafting (data not shown).

‡ Jensen UB, Jensen TG, Jensen PKA, Bolund L: Human keratinocytes transfected with growth factor genes in vitro form a multilayered tissue that can be transplanted onto nude mice: an approach to somatic gene therapy of skin ulcers (abstr). *J Invest Dermatol* 98:827, 1992.

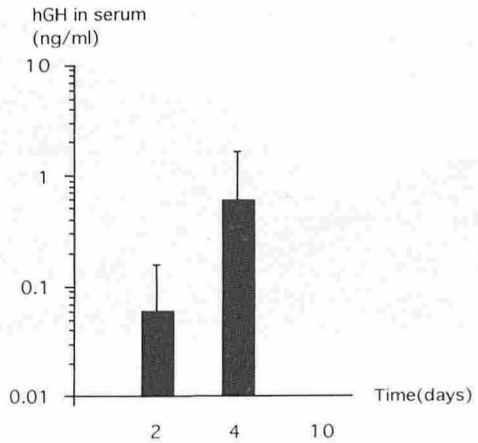

Figure 3. Human growth hormone in serum from nude mice transplanted with human primary keratinocytes after transfection with the hGH gene. Tail blood was placed on ice for 30 min and centrifuged for 10 min to obtain serum. HGH was measured by enzyme-linked immunosorbent assay. The columns represent the mean of five mice. *Error bars,* SD.

Figure 3 shows the amounts of hGH in serum from five mice. The variation among the mice was large. However in all mice hGH was detected after 2 d. In three mice the concentration of hGH was rising from day 2 to day 4. The highest amount detected in the serum was 2.6 ng/ml. At day 10 the levels were undetectable in all five mice. It should be noted that on day 5 the transplantation step 2 is performed, which removes the mouse tissue above the graft. Thus, it is possible that hGH was only secreted upwards to be absorbed by the mouse tissue above the graft. Another possibility might be that secretion through the basement membrane is abolished when the transfected cells are leaving the basement membrane to form differentiated cell layers as the tissue is maturing. To clarify the situation we used the *Escherichia coli* gene Lac Z to follow the transfected cells in a grafting experiment. Several cells staining positive for β-galactosidase were found at day 7, but exclusively in the stratum corneum **(Fig 4),** indicating that the transfected cells are rapidly lost from the basal cell layer.

DISCUSSION

Gene transfer to epidermal keratinocytes is of interest for many reasons. The cells are easily transfected to a high frequency [10], and the expression of the genes introduced is remarkably high. Epidermis is easily accessible and the fact that 30 kd molecules like Apo E are secreted through the basement membrane to the systemic circulation [4], and even larger molecules like albumin are transported into the epidermis [18], makes it a very tempting experimental system for somatic gene therapy.

In the present study we have used human primary keratinocytes to gain further insight into the possible application of somatic gene therapy on epidermal tissue. As shown in Fig 3 we find elevated amounts of hGH in the mouse serum on day 4 compared to day two. On day five, step 2 of the grafting technique is performed, which leaves the graft in its normal position as an integument. Five days later the amount of hGH is undetectable. Several explanations could account for these results. One possibility is that the expressed gene is rapidly shut down as has been the suggested explanation for the decreasing amount of factor IX observed by Gerrard *et al* [6]. Alternatively, it might be a reflection of the keratinocytes secreting hGH primarily upwards to be absorbed by the mouse tissue above the graft or it may be due to transfer of the transfected cells into the suprabasal layers, where the hGH is excluded from reaching and penetrating the basement membrane. To elucidate the problem we transplanted keratinocytes transfected with the Lac Z gene onto nude mice. Several blue cells were found after 7 d, but all were in the stratum corneum. None was found in the basal layer or the intermediate layers, supporting the idea that the transfected cells are rapidly

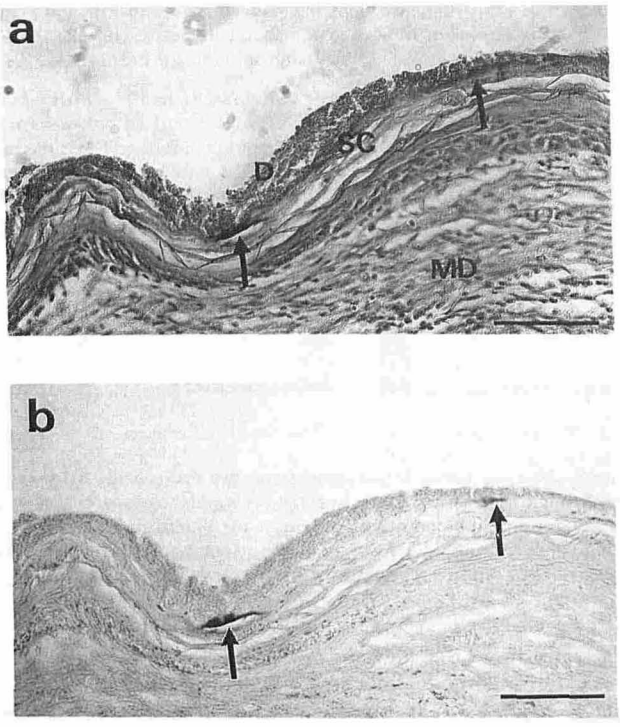

Figure 4. X-gal staining of human primary keratinocytes transfected with the Lac Z gene at day 7 post grafting (2 d after grafting step 2). *a)* Hematoxylin/eosin staining. *b)* Eosin staining. Two positive cells *(arrows)* are seen in the stratum corneum *(SC)*. Vaseline and debris from bandage *(D)*. Mouse "neo-dermis" *(MD)*. *Scale bar,* 100 μm.

lost from the basement membrane. The selective export of the transfected cells could be due to the very high expression of a foreign gene product, which might interfere with the programmed proliferation or differentiation of the keratinocyte. Alternatively, it is possible that the epidermal stem cells are refractory to gene transfer or become committed to differentiation upon transfection. Furthermore, it could be argued that the differentiation under *in vitro* conditions would induce loss of stem-cell function, and thus the probability of retaining competent transfected stem cells in the graft. However, because we are able to regenerate a well differentiated culture from the basal layer after several rounds of stripping off suprabasal cells in calcium free medium [19], we conclude that the short period in culture should not severely reduce the fraction of stem cells before grafting.

Despite the problems of not achieving long-term secretion into the blood stream we still find the epidermal tissue a promising system. However, we need to improve the system.

1) Steps have been taken towards prospective identification and separation of epidermal stem cells, e.g., based upon high expression of integrins [20]. Targeted transfection of such epidermal stem cells might make it possible to achieve a prolonged expression of the transfected genes in the basal cell layer.

2) Selection of the transfected primary keratinocytes during culture would ensure increased numbers of transfected basal cells. Because neomycin selection has been unsuccessful [5], attempts have been made in this direction by the use of the gene encoding L-histidinol dehydrogenase that renders dog keratinocytes resistant to L-histidinol [21]. So far we have not been able to adapt this method to human primary keratinocytes successfully.

3) A more stable expression of the introduced genes in the basal cell layer might be achieved by the use of an endogenous promoter (e.g., the keratin 14 promoter), which is exclusively active in the basal cells.

4) If inefficient secretion from the cells through the basement membrane turns out to be the problem, chimeric proteins may be constructed to improve this transport. Properties of the Apo E protein might be of interest in this context because this molecule has clearly been shown to be efficiently transported from the epidermis to the circulation [4].

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